AMENDMENTS TO THE SPECIFICATION

Please replace the paragraphs beginning at page 27, line 6 with the following paragraphs:

~~Figure 11~~ Figure 10 illustrates the DNA blot hybridisation analyses of genomic DNA from non-transformed (CON 1) and transgenic red fescue. DNA samples of 10-30 μg were restricted with *Hin*DIII (A) or *Eco*RI (B) and probed with a 0.4-kb fragment containing the 3' -end of the ubiquitin intron and the 5' -end of the *LpTFL1* coding region (MS56-LP4REV, see Table 1). *Hin*DIII release from pLPTFL1 a 2.8-kb fragment containing the entire *LpTFL1* cassette (arrowhead). *Eco*R1 has a single restriction site on pLPTFL1, which is a 5.5 -kb plasmid (arrowhead);

~~Figure 12~~ Figure 11 illustrates the transgene levels and phenotypes of the transgenic UBI::*LpTFL1* fescue lines. A. Average number of spikes produced per clone during the first (grey bars) and the second (checked bars) season by the lines (A-N), the non-transformed (CON), and the transformation controls (BAR), compared with the relative levels of *LpTFL1* mRNA (black bars, second Y-axis). The white bar represents the level of a transcript corresponding to a truncated *LpTFL1* mRNA. B. The average stem length of each line measured during the first season (grey bars) and compared with *LpTFL1* mRNA levels (black bars, second Y-axis). Error-bars show the standard deviation from the average value within each line. All *LpTFL1* mRNA levels are relative to the level of *LpACTIN*, and the highest detected value was set to 100 (line J).

~~Figure 13~~ Figure 12 illustrates an RNA gel blot analysis of primary transformants from different UBI::*LpTFL1* lines. 2.5μg of poly-A^+ mRNA each line were blotted and probed with a

200 bp *LpTFL1* or a 450 bp LpAC*TIN* cDNA probe. All the lines included were positive for the transgene as was verified by PCR (lower panel) using the primers MS56 and Lp4REV which amplifies a 425 bp fragment corresponding to the last 75 bases in the ubiquitin 3' end and 350 bases of the *LpTFL1* cDNA;

~~Figure 14~~ Figure 13 illustrates the flowering and non-flowering plants of 400 days old fescue wild-type (CON), transgenic controls (BAR) and UBI::*LpTFL1* lines (A-M). Each line represents a single transformation event. Black bars below each picture shows to the *LpTFL1* transcript level relative to the level in the non-transformed control plants, which was set to 1.0. The white bar indicates that the level corresponds to level of an overexpressed truncated *LpTFL1* transcript; and

~~Figure 15~~ Figure 14 illustrates the Panicle phenotypes of red fescue wild-type (CON1) and of the UBI::*LpTFL1* transgenic lines A and C, which overexpress a truncated and a correct *LpTFL1* transcript, respectively. Bar = 1 cm.

~~Figure 16~~ Figure 15 is a table showing the transformation efficiency and the floral activity for a number of transgenic ryegrass lines transformed with *LpTFL1*.

~~Figure 17~~ Figure 16 shows analysis of transgene integration by PCR in UBI::*LpTFL1* transgenic red fescue lines.

Please replace the paragraph beginning at page 42, line 9 with the following paragraph:

Following three months of vernalization, all transgenic lines (inclusive all the lines which were tested negative for presence of the *LpTFL1* transgene and the non-transformed control) were transferred to LD conditions for floral induction. The number of inflorescences varied

among the transformed lines with the biggest variation observed between the cultivars. This variance was also observed in the co-transformation efficiency (~~Figure 16~~Figure 15) and reflects how different the cultivars responded to the transformation event. 'F6' gave the highest co-transformation efficiency (78%) followed by 'ACTION' (75%) and 'TELSTAR' (54%). Among the plants, which were tested negative for the *LpTFL1* transgene, 'F6' also produced more flowers (12,3 ± 5,1) than the two other cultivars ('TELSTAR'; 6,0 ± 4,2) and ('ACTION'; 1,4 ± 1,2). 'TELSTAR' and 'F6' in general looked more vigorous than did the cultivar 'ACTION'.

Please replace the paragraph beginning at page 42, line 24 with the following paragraph:

Using real-time RT-PCR we tested, whether the reduction in inflorescence production was correlated with the level of expression of *LpTFL1* from the *UBI::LpTFL1* transgene. We could detect *LpTFL1* transgene expression in 16 of the 22 PCR positive lines ~~(Fig. 10)~~. In order to distinguish between the transgene and the endogenous *LpTFL1* a NOS terminator primer was used in combination with and internal *LpTFL1* primer in the real-time RT-PCR. Subsequent analysis showed that the endogenous *LpTFL1* mRNA level in the leaves was 100-fold less than the lowest detected *LpTFL1* transgene mRNA level at the point of harvest (not shown). A very high transgene expression level was detected in several lines, and we observed a clear and very dramatic effect of the *UBI::LpTFL1* transgene when *LpTFL1* was expressed at high levels. Five of the six lines (31, 32, 34, 35, 36) in which we detected the highest *LpTFL1* expression did not flower, and nine lines (23, 26, 27, 29, 31, 32, 34, 35, 36) of the 16 *LpTFL1* overexpressing lines

remained non-flowering throughout the season. Overexpression of *LpTFL1* did not cause any other morphological changes when compared to the wild-type.

Please replace the paragraphs beginning at page 43, line 18 with the following paragraphs:

The control of floral transition (ie. the transition from vegetative to reproductive growth) has been studied extensively, especially in *Arabidopsis*, and a number of key regulators have been identified (for a recent review, see Simpson *et al.* (1999)). Knowledge of the biological function of these regulators is derived either from mutant studies or from experiments in which these genes were constitutively expressed in annual plants such as *Arabidopsis*, *Antirrhinum*, or tobacco. Previous results showed that *LpTFL1* is a strong repressor of flowering in annual plants, such as *Arabidopsis* (Jensen *et al.*, 2001). By introducing *LpTFL1* into ryegrass under the control of the maize ubiquitin promoter, we tested whether constitutive expression of *LpTFL1* was capable of preventing or inhibiting flowering in a perennial plant. Thirty six transgenic lines were produced of which 22 were tested positive for the *LpTFL1* transgene. Flowering was markedly reduced among the PCR positive plants, and ten lines (45%) remained non-flowering during the flowering season. In contrast, only two lines out of the 14 PCR negative lines (14%) were non-flowering ~~(Fig. 10)~~.

The level of *LpTFL1* expression was tightly linked to the control of the vegetative to the reproductive phase. However, there was no linear correlation between the level of transgene expression and the flowering time (heading date) as previously observed in *Arabidopsis* (Jensen

et. al., 2001), and the floral repression was more seen as reduction in inflorescence production as a delay in heading date. We could detect *LpTFL1* transgene mRNA in 16 of the 22 PCR positive lines ~~(Fig. 10)~~, and nine of these lines (56%) remained non-flowering. Expression of *LpTFL1* at high levels comparable to housekeeping genes such as *GAPDH*, in this case prevented heading in five out of six lines (~~Fig. 10,~~ line 31-36). No meristem proliferation or stem elongation was observed in the non-heading lines, which indicates that the plants were arrested in the vegetative phase.

Please replace the paragraph beginning at page 44, line 22 with the following paragraph:

The effect of *LpTFL1* overexpression was independent of genotype. Three different genotypes were used in the experiment and even though they all responded differently to the transformation with respect to co-transformation efficiency (~~Figure 16~~Figure 15), the percentage of non-flowering *LpTFL1* overexpressing lines were equally distributed among them; ACTION, 55%; TELSTAR, 50%; and F6, 60%.

Please replace the paragraph beginning at page 46, line 15 with the following paragraph:

Genomic DNA was isolated from leaves of primary transformants (T0 generation) by the FastDNA® ORANGE kit DNA isolation system (Bio 101), and the presence of the transgene was determined by PCR. Different primer combinations were used to examine the genomic integration and arrangement of the transgenic DNA (~~Figure 17~~Figure 16).

Please replace the paragraph beginning at page 46, line 24 with the following paragraph:

Genomic DNA for the gel blot analysis was isolated from the leaves of one to three individuals of different transgenic lines by the Phytopure® Genomic DNA isolation system (Nucleon). DNA (10-30 μg) were digested overnight with restriction endonucleases *Hin*DIII and *Eco*RI (separately) and fractionated on a 0.8% agarose gel and blotted onto Amersham Hybond N membrane in 20% SSC according to the manufacturer's recommendations. Probe DNA generated by PCR using the primer set MS56-LP4REV on plasmid DNA (~~Figure 17~~Figure 16) was radiolabeled with γ-^{32}P-labelled dCTP (3,000 Ci/mmol) through the random primer method (Megaprime, Amersham). Pre-hybridisation, hybridisation and the subsequent washing steps were performed according to standard protocols.

Please replace the paragraphs beginning at page 47, line 17 with the following paragraphs:

Eighteen transgenic fescue lines were obtained by microprojectile bombardment. In addition, two lines (BAR1 and BAR2) were obtained by transformation only with the plasmid pAHC20. All lines were resistant to BASTA® and showed phosphinothricin acetyl transferase activity. Plants regenerated from a single transgenic callus (generation T_0) were designated as a "transgenic line". Thus, each transgenic line traced back to a different tissue culture and represented an independent transformation event. PCR analyses of transgenic fescue leaf DNA

using the primers MS56 and LP4REV (~~Figure 17~~Figure 16) indicated that *LpTFL1* was present in 14 lines, giving a 77% co-transformation efficiency. These 14 lines (A-N) together with BAR1, BAR2 and two non-transformed lines were selected for further characterisation.

Transgene Integration

The DNA from the transgenic plants was digested with *Hin*DIII, which released a 2.8-kb fragment containing the ubiquitin promoter and the *LpTFL1* coding region (~~Figure 17~~Figure 16). Restriction patterns of transgenic DNA were complex in several lines (~~Fig. 11~~Fig. 10). Restriction fragments of the expected size were found in four lines (D, I, J, and L, ~~Fig. 11A~~ Fig. 10A and not shown). All lines contained fragments larger or smaller than the expected size, which represented rearrangements of the transgene DNA. There were no rearranged fragments of the same size recurrently observed in different lines (~~Fig. 11A~~Fig. 10A) except for a 2.1 -kb fragment, which was also present in the controls and may correspond to the endogenous *F. rubra TFL1*-like *(FrTFL1)* gene. Faint or smeared signals were also detected in restricted DNA from BAR1 and BAR2 (~~Fig. 11~~Fig. 10), which may represent the plasmid pAHC20 that carries the same ubiquitin promoter:exon:intron construct to drive *Bar* expression.

DNA from transgenic plants was also digested with *Eco*RI which has only one restriction site in the vector at the 3'-end of the NOS terminator and was expected to yield fragments corresponding in size to the repeats in a pALPTFL1 concatamer if plasmid concatenation had occurred. Multiple different-sized *Eco*RI restriction fragments hybridising to the *intron::LpTFL1* probe (~~Fig. 11B~~Fig. 10A) indicated that concatenation of full-length plasmid copies was not the predominant mode of transgene organisation in the plant genome. Two lines (D and I) contained

fragments of the expected size (5.5 kb), however the subsequent attempt to PCR amplify the transgene promoter in these lines failed (see below). It was difficult to determine the exact transgene copy number especially because several lines contained truncated plasmid copies. Nevertheless, we estimated it to vary from two (line G) to twenty (line D).

Long PCR using different primer combinations to amplify parts of the ubiquitin-exon-intron-LpTFL1-nos cassette (see ~~Figure 17~~ Figure 16) was performed to examine if the transgenic lines contained intact cassettes or if transgene rearrangement had occurred internally in this region. The 3'-end of the cassette containing the *LpTFL1* coding region and the NOS terminator appeared to be intact in all lines except for A and F (~~Figure 17~~Figure 16). In addition to the fragments of expected size (0.6 kb), a 0.5 kb fragment was detected in three lines D, G and N, when PCR was performed with the primer set MS56 and LP575. When PCR was performed with the primers LP0 and MS8 we detected fragments larger than the expected size in lines D, I and N. Such fragments may be amplified by each of the single primers if the transgene had integrated into the genome in a tail-to-tail manner. Alternatively, fragmented transgene DNA may have been dispersed in the fescue genome. Smaller fragments must reflect DNA deletions, and since these fragments were only detected when using the primers MS56-Lp4REV and not LP0-MS8, the deletion is likely located in the 3'-end of the ubiquitin intron.

The promoter part of the UBI::LpTFL1 cassette was analysed by PCR using two primers (MS33 and MS31) located 500 and 100 bp upstream the TATA box, respectively, in combination with primers matching the LpTFL1 coding region. The results schematically described in ~~Figure 17~~Figure 16, revealed that two lines (J and L) contained the full-length promoter, while 6 lines only contained a short partial ubiquitin promoter (including the MS31 primer site). In line B, C,

E, F, I and K the promoter part was either absent or dispersed and/or reoriented from the LpTFL1 coding region, and in line D and N a 1.5-kb DNA fragment had been deleted between MS33 and LP4REV (including MS31 and the TATA box). A 100 bp deletion was also found in the promoter of lines G, and although the exact location was not determined we assume it to be close to the 3'-end of the UBI intron. The intron part was found to be intact in line A, D, G, H, J, L and M but not in line B, C, E, F, I, K and N (MS31-LP4REV). In all, two lines (J and L) were found to contain at least one complete expression cassette (Ubi-ex-intron-LpTFL1-nos).

Please replace the paragraphs beginning at page 50, line 3 with the following paragraphs:

The number of inflorescences produced by each clone during the first season varied markedly between the lines (from 0 to 138, ~~Figure 12A~~ Figure 11A). Fewer inflorescences were produced the second year because the clones were divided into smaller units. Stem (culm) length also varied between the lines ~~(Fig. 10B)~~, and it did not change significantly from the first to the second flowering season (not shown). Four UBI::LpTFL1 lines (K, L, M and N, ~~Figure 12~~ Figure 11A and B) did not flower during the seven months following the first vernalization, and three of these lines (K, L, and M) also remained non-flowering during the second season. Two lines (I and J) produced only a single flower from three individual clones during the first season and only one and three flowers during the second season, respectively.

RNA gel blot analysis was performed to test whether the reduction and delay in inflorescence production was correlated with the expression of LpTFL1 from the UBI::LpTFL1

transgene. The level of LpTFL1 message varied from zero to levels comparable to ACTIN mRNA (~~Fig. 13~~Fig. 12). Three of the four lines (K, L, M) in which the highest LpTFL1 expression was detected did not flower, and the fourth line (J) produced only 0.3 inflorescence per clone (~~Fig 12A and 14~~Fig. 11A and 13). Lines with a lower level of LpTFL1 message produced flowers, and there was a trend (although not statistical significant with the present material) towards a reduction in the number of inflorescences per clone with increasing levels of LpTFL1 mRNA (~~Fig. 12A~~Fig. 11A).

No LpTFL1 message was detected in line D, F and N (~~Fig. 12 and 13~~Fig. 11 and 12). This finding correlated well with the observation that the transgenes in these lines either lacked the UBI promoter (line F) or had a partial UBI promoter lacking the TATA box (line D and N, ~~Figure 17~~Figure 16).

The LpTFL1 message in line A was 80-120 bp smaller than expected (~~Fig 13~~Fig. 12). We propose that this fragment represents a truncated LpTFL1 transcript, which is overexpressed in this line. This assumption is strengthened by the fact that, for this line, we were unable to PCR amplify the LP0-MS8 fragment, which contains the LpTFL1 coding region and the NOS terminator (~~Figure 17~~Figure 16). In addition, we found that plants from line A flowered simultaneously with the wild-type and produced the highest average number of inflorescences among all the UBI::LpTFL1 lines (~~Fig. 12A~~Fig. 11A). Line A plants were also among the tallest plants included in the investigation (~~Fig 12B~~Fig. 11B), and they produced panicles, which were generally reduced in size compared to the control (~~Fig. 15~~Fig 14). Oppositely, the single flowering plant of the high expressing line J, was the shortest of all flowering plants (~~Fig.~~

~~12A~~Fig 11A). However, with the present data in hand, there is no statistical significance to confirm a correlation between the level of LpTFL1 expression and culm length.

A. DISCUSSION

Eighteen Basta® resistant red fescue lines were obtained by particle bombardment. Plants from fourteen different lines were tested positive for the gene of interest by PCR. DNA gel blot analysis of different lines revealed that the transgene had integrated into the fescue genome in a complex fashion and that multiple transgene rearrangements had occurred. Transgene rearrangements included deletions in the promoter regions and in the LpTFL1 gene (~~Figure 17~~Figure 16). Highest expression of LpTFL1 was detected in plants containing the full UBI::LpTFL1 cassette. Deletion of promoter sequence lead in most instances to a reduction in LpTFL1 expression compared to the high expressing lines (~~Figure 17 and Fig. 12~~Figure 16 and Fig. 11). Expectedly, if the deletion included the TATA box, the partial promoter was defect and no LpTFL1 transcripts could be detected in the plants. However, one line (K) expressed LpTFL1 at high levels although we could not PCR amplify any fragments corresponding to the UBI promoter construct in this line. There is no obvious explanation for this observation, but either none of the three PCR reactions worked or, alternatively, parts of the cassette could have integrated into a transcriptionally active region.

Analysis of the transgenic lines showed that LpTFL1 expression in red fescue is tightly linked to the control of vegetative to reproductive phase shift. All lines containing the LpTFL1

transgene, except line A (discussed below), flowered at least two weeks later than the wild-type and the BAR controls. However, there was no linear correlation between the level of transgene expression and flowering time (heading date) as was previously observed in Arabidopsis (Jensen et al., 2001). Expression of LpTFL1 at high levels comparable to housekeeping genes such as ACTIN, in this case prevented heading in three out of four lines and in the fourth line only one inflorescence was produced within three clones (~~Fig. 12A and 14~~Fig. 11A and 13). At moderate expression levels, LpTFL1 expression caused a general reduction in the number of inflorescences and in the stem length but an increase in panicle branching, although the statistical significance of these observation requires a more thorough investigation of the second generation plants. . Similar observations were made by Nakagawa et al. (2002) in the analysis of transgenic rice overexpressing RCN1/2. They found that constitutive expression of RCN1/2 at moderate levels were associated with a three-fold increase of secondary branches and even production of tertiary branches, which is not seen in wild-type rice. Expression of RCN1/2 at high levels led to stem retardation and a 'never-heading' phenotype. However, the 'never-heading' plants still produced a flag leaf and an immature panicle, enclosed by leaves, indicating that the transition from vegetative to reproductive phase finally took place (Nakagawa et al., 2002). These results are in contrast to our observations, which show that the 'never-heading' red fescue plants presented here, are arrested in the vegetative phase, since they do not produce stems or panicles.

Please replace the paragraphs beginning at page 53, line 1 with the following paragraphs:

Despite the fact that no LpTFL1 expression was detected in line N, these plants remained non-flowering during the first season. It is most likely that this deviation from the other results can be ascribed to the carry-over effects from the tissue culture, since this line started to produce flowers during the second season (~~Fig. 12A~~Fig. 11A).

The phenotype of the transgenic line A was reminiscent of a putative weak Festuca rubra tfl1-like(frtfl1) mutant phenotype. In this line, culm and panicle formation was favoured at the expense of decreased leaf production (~~Fig. 14~~Fig. 13), and the panicles were more compressed and wrinkled than the wild-type (~~Fig. 15~~Fig. 14). Line C plants in contrast, produced panicles, which were generally larger and contained more spikes with more spikelets than the wild-type panicle. Line C plants expressed LpTFL1 at a relatively high level, suggesting that the increased branching is a direct effect of increased levels of LpTFL1. Consistent with this hypothesis is the assumption that the decreased branching observed in line A is caused by a C-terminal truncation of the LpTFL1 protein. Interestingly, it was recently found that the function of the proteins belonging to the TFL1 family in Arabidopsis is dependent on the C-terminal part of the protein. FLOWERING LOCUS T (FT), which is very homologous to TFL1, but acts oppositely (Kardailsky et al., 1999), is mainly determined by the C-terminal part of the protein. By swapping exons between the FT and TFL1 cDNAs, Ahn and Weigel, (2001) found that the last exon distinguishes between FT- and TFL1-like properties of the chimeric gene.

Please replace the paragraph beginning at page 54, line 5 with the following paragraph:

Our results show that expression of the heterologous LpTFL1 in red fescue at high levels can prevent flowering (~~Fig. 12A and 14~~Fig. 11A and 12). Additionally, it appears that the level of LpTFL1 expression in flowering plants may cause a reduction in culm length (~~Fig. 12B~~Fig. 11A) and leaf width (not shown), although this needs to be further examined. No other morphological effects of the transgene expression were observed.